Intra-patient Heterogeneity of *BRAF* and *NRAS* Molecular Alterations in Primary Melanoma and Metastases

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Mutations in *MAPK* signalling genes are driver events in melanoma, and have therapeutic relevance in the metastatic and adjuvant setting. This study evaluated the intra-patient heterogeneity of *BRAF*, *NRAS* and *c-KIT* mutational status between 30 primary melanomas and 39 related metastases, using molecular analysis and immunohistochemistry. *BRAF* mutations were identified in 46.7% of primary melanomas and 48.7% of metastases and *NRAS* mutations in 20% and 25.6%, respectively. Intra-patient heterogeneity was detected in 13.3% of patients for both *BRAF* and *NRAS* genes and was not associated with clinico-pathological characteristics of melanomas or metastases. High consistency was observed between immunostaining and molecular methods for $BRAF^{V600E}$ (k = 0.90; $p < 0.001$) and $NRAS^{Q61R}$ (k = 0.87; $p < 0.001$). These findings demonstrate a relevant intra-patient heterogeneity between primary and metastatic lesions that is independent of clinical variables and methodological approach.

Key words: melanoma; metastases; *BRAF*; *NRAS*; *c-KIT*; heterogeneity.

Accepted Nov 27, 2019; Epub ahead of print Nov 27, 2019

Acta Derm Venereol 2020; 100: adv00040.

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SIGNIFICANCE

Cutaneous melanoma is one of the most aggressive and treatment-resistant tumours. Intra-patient concordance of mutations in genes, such as *BRAF*, *NRAS* and *c-KIT*, between primary melanoma and related metastases is a critical aspect that has become even more relevant with the introduction of therapies targeting specific mutations. This study evaluated the intra-patient heterogeneity of *BRAF*, *NRAS* and *c-KIT* mutational status in 30 primary melanomas and 39 related metastases, using molecular analysis and immunohistochemistry. Clinically meaningful intra-patient heterogeneity was found between primary melanoma and related metastases, independent of the technical approach, thus supporting the polyclonal model of melanoma progression.

Melanoma is considered one of the most aggressive cancers, with an increasing incidence worldwide over recent decades (1). The treatment of metastatic disease has been a challenge in the recent past, with a low survival rate of approximately 20% at 5 years for stage IV patients (2–5). Nevertheless, the introduction of new therapeutic approaches, such as targeted therapy and immunotherapy, based on a growing understanding of molecular alterations involved in melanoma pathogenesis, has significantly improved outcomes for patients (6).

The most relevant molecular pathway implicated in melanoma pathogenesis is the mitogen-activated protein kinase (MAPK) cascade, which is dysregulated in approximately 80% of melanomas (7). Within this pathway, mutations in *BRAF*, *NRAS* and *c-KIT* are considered driver events and have a strong clinical relevance for melanoma treatment (8–10). Activating *BRAF* mutations occur in approximately 50% of cutaneous melanomas, mainly at codon 600 in exon 15, with the most common mutation being the V600E change (identified in approximately 80% of cases) (8). Oncogenic *NRAS* mutations are found in 15–25% of cutaneous melanomas and are usually detected at codon 61, mainly with a glutamine to arginine/lysine/leucine substitution (Q61R/K/L) (8). Finally, approximately 3% of all melanomas carry somatic mutations in exons 11 (L576P) and 13 (K642E) of the *c-KIT* gene, but additional *c-KIT* aberrations might include mutations in exon 17 and gene amplifications (8). Mutations of *BRAF*, *NRAS* and *c-KIT* are usually identified by molecular methods (11), but immunohistochemical (IHC) analysis represents a useful option, being widely available, less labour-intensive and less expensive (11–14). However, there is no consensus so far on the best testing method (11).

Mutational concordance between primary melanoma and related metastases is a critical aspect that has become even more relevant with the introduction of therapies targeting specific mutations of driver genes (15). Intra-patient molecular heterogeneity between primary and metastatic lesions may exist, and changes in mutational pattern might occur during progression (16). Data on heterogeneity have been reported mainly for the *BRAF* gene (17–26) and found in 13–15% of patients in 2 recent meta-analyses, with different rates depending on the type of tested metastatic tissue (16, 27). However, differences across the studies could also reflect technical issues, since

higher heterogeneity rates have been reported for molecular approaches than for IHC-based methods (28). Few studies have focused on the intra-patient concordance of *NRAS* mutations between primary and metastatic lesions, with percentages of discordance ranging from 0 to 9% (17, 24, 26, 29). No data are available on *c-KIT* mutational heterogeneity in cutaneous melanoma.

This study aimed to investigate the mutational status of *BRAF*, *NRAS* and *c-KIT* genes in patients with metastatic melanoma in order to evaluate the intra-patient molecular heterogeneity between primary tumour and related metastases and to compare the consistency of mutational findings obtained by molecular and IHC analyses.

MATERIALS AND METHODS

Patients' and tissue samples

Patients with a histopathologically confirmed diagnosis of metastatic melanoma were recruited between January 2012 and December 2017 at the Department of Dermatology, University of L'Aquila, Italy. For each patient, tissues were retrieved from the primary melanoma and at least one metastasis. Patient's clinical information including sex, age at diagnosis, anatomical site of primary melanoma and related metastases and AJCC stage 8th edition (30) were collected.

Haematoxylin-eosin-stained sections of primary and metastatic tissues were reviewed by 2 experienced pathologists (MDP and GC) to confirm the diagnosis. The following clinico-pathological features of primary tumours and/or metastasis were collected: anatomical location, histopathological variant, Breslow thickness, presence of ulceration, number of mitoses/mm^2, presence of melanoma-associated naevus and CSD (histologically defined according to the degree of solar elastosis).

Approval for this study was obtained from the Ethics Committee of the ASL-01 Avezzano, Sulmona, L'Aquila (protocol number 0012038/11). Written informed consent was provided by all patients. The study was performed according to the principles of the Declaration of Helsinki.

Molecular analysis

Somatic DNA was extracted from 5 formalin-fixed paraffin-embedded (FFPE) tissue sections (each 10-micron in thickness) obtained from the same tissue block used for IHC, by microdissection of marked tumour-rich areas from primary melanoma and metastases using a QIAmp DNA Micro tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Exon 15 of *BRAF*, exon 2 of *NRAS*, and exons 11, 13 and 17 of c-*KIT* were screened by Sanger sequencing. Briefly, PCR amplification of the regions of interest was performed in a Simply-Amp PCR-System (Thermo-Fisher, Foster City, CA, USA) using primers listed in Table SI[1]. PCR experiments were performed as described previously (14). Amplicons were sequenced on 3500 GeneticAnalyzer (Thermo-Fisher). The variants were detected using the Applied Biosystems Minor Variant Q11 Finder software version 1.0 (Thermo Fisher), specific to calling low-frequency somatic variants. Competitive allele-specific TaqMan™ PCR (castPCR™ Technology) assays were used to confirm sequencing results in *BRAF* and *NRAS* wild-type samples and if discrepancies were found between molecular analysis and IHC. PCRs containing 20 ng DNA, 1X TaqMan™ Mutation Detection Assays (BRAF^{V600E}, Hs00000111_mu; BRAF^{V600K}, Hs000000002_rm; NRASQ^{61R}, Hs00000808_mu; NRAS^{Q61L}, Hs00000807_mu; NRAS^{Q61K}, Hs00000804_mu; NRAs^{Q61}H, Hs00000809_mu), 1X TaqMan™ Genotyping Master Mix (Thermo-Fisher) and water to reach a final volume of 20 µl were performed using the standard TaqMan protocol on a 7500 Fast Real Time-PCR System (Thermo-Fisher). Positive and negative controls were used for experiments of mutation detection.

c-KIT copy number was assessed by quantitative real-time PCR for exon 13 and compared with *GAPDH* as an internal control, as described previously (29). Briefly, PCR reactions were performed using PowerUP™ SYBR™ Green Master Mix (Thermo-Fisher), with a 20 µl total volume and 50 ng genomic DNA on a 7500 Fast Real Time-PCR System. Primers for *c-KIT* exon 13 and *GAPDH* are listed in Table SI[1]. The thermal cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. Each gene was analysed in triplicate. Samples that did not amplify by 35 cycles were considered to be of insufficient quality and were excluded from the analysis. For each sample, ΔCt for *c-KIT* vs. *GAPDH* was calculated as ΔCt = Ct (*c-KIT*) – Ct (*GAPDH*) and then calibrated to individual reference genomic DNAs from 3 normal skin tissue samples and confirmed by calibration to a commercial human genomic reference DNA (Thermo-Fisher). Relative copy number evaluation was performed by the comparative $2^{-\Delta\Delta Ct}$ method and converted to absolute copy number by assigning a value of 2 to the reference DNA.

Immunohistochemistry

IHC was performed on FFPE tissue sections of 4 µm thickness. The presence of *BRAF*^{V600E} and *NRAS*^{Q61R} mutants and c-KIT expression were evaluated using the following monoclonal antibodies: *BRAF*^{V600E} VE1 clone (Spring Bioscience, Pleasanton, CA, USA), *NRAS*^{Q61R} SP174 clone (Spring Bioscience), and CD117/c-Kit polyclonal (Spring Bioscience) at a dilution of 1:30, 1:80 and 1:100, respectively. Sections were freshly cut, dried at 60°C for 30 min, deparaffinized and rehydrated. Immunoreactions were performed on Ventana BenchMark GX automatic immune stainer (Ventana Medical Systems Inc., Tucson, AZ, USA) using the Ultra View Universal Alkalin Phosphatase Red Detection Kit, as previously reported (13). No chromogen was detected when primary antibody was omitted. Positive controls were mounted on each section subjected to immunostaining. Negative controls were included in each run.

The evaluation of IHC status was performed independently by 2 observers (MDP and GC) blinded to the molecular mutational status; disagreement was resolved by consensus. Cytoplasmic staining of *BRAF*^{V600E} VE1, *NRAS*^{Q61R} SP174 and CD117/c-Kit polyclonal antibodies in melanoma cells was considered as positive or negative and graded for intensity, according to previously published criteria (14, 31). Staining for *BRAF*^{V600E} VE1 and *NRAS*^{Q61R} SP174 was considered positive if the percentage of stained melanoma cells was more than 10% and classified as homogeneous (staining in >95% of cells) or heterogeneous (staining in <95% of cells) (14). Negative staining was defined either as absence of any cytoplasmic labelling or staining of single interspersed melanoma cells (<10%). Intensity of staining was graded as weak, moderate or strong (14). CD117/c-Kit staining was assessed on the percentage of stained cells and strength of staining: 0, no staining; 1+, weak staining in isolated groups of melanoma cells; 2+, weak and widespread staining in <50% of melanoma cells; 3+, moderate staining in 50–75% of melanoma cells; 4+, strong staining in >75% of melanoma cells. Staining was classified as positive in the presence of moderate/strong membranous and cytoplasmic staining (3+/4+) and as negative if there was absence of cytoplasmic staining and if staining was weak and widespread (0/1+/2+).

[1]https://doi.org/10.2340/00015555-3382

Statistical analysis

For statistical analysis, categorical variables for primary melanoma were grouped as follows: anatomical site (head/neck, trunk, extremities, acral), histopathological subtype (superficial spreading melanoma [SSM], nodular melanoma), chronic sun damage (CSD or no CSD), presence of ulceration (yes, no), number of mitoses/mm^2 and presence of melanoma-associated naevus (yes, no). Synchronicity (defined as a metastasis diagnosed at the same time as the primary melanoma) and anatomical site (skin, lymph node, visceral, brain) were recorded for metastases.

Semi-quantitative data (age at diagnosis, Breslow thickness) were analysed by means of Student's *t*-test or by medians with Mann–Whitney test. Univariate analysis by χ^2 test or by Fisher's exact test was used to test the significance of mutation frequency according to clinico-pathological characteristics of melanoma patients and tumours. Molecular findings were used as the gold standard for statistical analysis. Cohen's k coefficient test was used to measure the agreement between molecular and IHC methods in determining *BRAF* and *NRAS* mutational status. Samples harbouring the *BRAF*^{V600K} mutation according to molecular analysis, that were wild-type on IHC VE1 staining, were not considered for Cohen's k analysis. *p*-values less than 0.05 were considered statistically significant. Statistical analysis was performed using the statistical package SPSS 17.0 (SPSS Incorporated, Chicago, USA).

RESULTS

Patients' and tumour samples

Overall, a total of 69 tumour tissues (30 primary melanomas and 39 related metastatic lesions) were collected from 30 patients with advanced melanoma (25 stage III and 5 stage IV). Enrolled patients included 17 males and 13 females, with a median age at first diagnosis of 65 years (range 25–84 years). In detail, 23 patients were diagnosed with 1 metastasis, 5 with 2 metastases and 2 with 3 metastases. Demographic and clinical characteristics of patients and tumour tissues are reported in **Table I**.

Twelve primary melanomas (12/30, 40.0%) were located on the extremities, 10 on the trunk (10/30, 33.3%), 5 (5/30, 16.7%) on the head/neck region and 3 (3/30, 10.0%) on acral sites. The majority of tumours were of the nodular histological subtype (23/30, 76.7%) followed by SSM (7/30, 23.3%); no acral lentiginous or lentigo maligna melanomas were diagnosed in our group of patients. Median Breslow thickness was 4.2 mm (range 0.6–30 mm). A pre-existing melanocytic naevus was associated in 36.7% (11/30) of primary melanomas.

The majority of metastatic tissues were collected from lymph nodes (23/39, 59.0%), followed by skin (11/39, 28.2%), brain (2/39, 5.1%), colon (1/39, 2.6%), liver (1/39, 2.6%) and parathyroid gland (1/39, 2.6%). Median time to first metastasis was 3 months (range 0–51 months). Metastases were synchronous in 17 (17/30, 56.7%) patients and metachronous in 9 (9/30, 30.0%); 4 (4/30, 13.3%) patients had both synchronous and metachronous metastases.

Molecular analysis

The distribution of *BRAF*, *NRAS* and *c-KIT* mutations in all analysed tissues is shown in **Table II**. *BRAF* mutations at codon 600 were detected in 33 of 69 (47.8%) tumour tissues, with 23 harbouring the *BRAF*^{V600E} (23/33, 69.7%) and 10 the *BRAF*^{V600K} (10/33, 30.3%) mutation. Distribution of *BRAF* mutations was similar between primary melanomas (14/30, 46.7%) and metastatic samples (19/39, 48.7%) ($p=0.42$). Sixteen of 69 tumour tissues (16/69, 23.2%) carried *NRAS* mutations at codon 61 with the following genotypes: *NRAS*^{Q61R} (8/16, 50.0%), *NRAS*^{Q61L} (7/16, 43.7%) and *NRAS*^{Q61K} (1/16, 6.3%). Among the 16 *NRAS* mutated tumours, 6 were primary melanomas (6/30, 20.0%) and 10 (10/39, 25.6%) metastatic samples ($p=0.61$). Finally, only 1 missense *c-KIT* mutation, L802F mutation in exon 17, was detected in one (1/69, 1.4%) primary melanoma diagnosed on the upper extremity. A mutation in at least 1 of the 3 genes was present in 21 of 30 (70.0%) primary melanomas and 29 of 39 (74.3%) metastatic samples, for a total of 72.5% tissues (50/69). All tissues were mutually exclusive for *BRAF*, *NRAS* and *c-KIT* mutations (Table II). *c-KIT* gene amplification was carried out in 65/69 (94.2%) samples with 4 samples missing due to lack of DNA. An increased copy number (≥3 copies) was identified in 3 (3/69 4.3%) samples, with 2 of them being primary melanomas with a high level of chronic sun damage (CSD). All cases with *c-KIT* amplification carried concomitant *BRAF*^{V600}

Table I. Demographic and clinical characteristics of melanoma patients and histopathological features of tumours and metastasis

Characteristics of patients (*n* = 30)	
Sex, *n* (%)	
Males	17 (56.7)
Females	13 (43.3)
Age, years, median (range)	65 (25–84)
Metastases, *n* (%)	
2	5 (16.6)
3	2 (6.7)
Synchronous/metachronous, *n* (%)	
Synchronous	17 (56.7)
Metachronous	9 (30.0)
Stage, *n* (%)	
III[a]	25 (83.3)
IV	5 (16.7)
Characteristics of primary melanoma (*n* = 30)	
Breslow thickness, median (range)	4.2 (0.6–30)
Histopathological subtype, *n* (%)	
Superficial spreading melanoma	7 (23.3)
Nodular melanoma	23 (76.7)
Anatomical site, *n* (%)	
Head/neck	5 (16.7)
Trunk	10 (33.3)
Extremities	12 (40.0)
Acral	3 (10.0)
Associated naevus, *n* (%)	
Yes	11 (36.7)
No	19 (63.3)
Ulceration, *n* (%)	
Yes	20 (66.7)
No	10 (33.3)
Solar elastosis, *n* (%)	
Yes	18 (60.0)
No	12 (40.0)
Characteristics of metastases (*n* = 39)	
Anatomical site, *n* (%)	
Lymph nodes	23 (59.0)
Skin	11 (28.2)
Brain	2 (5.1)
Visceral	3 (7.7)

[a]Stage III patients included 20 patients with nodal metastases and 5 patients with in-transit metastases without nodal involvement.

Table II. Summary of mutational patterns in primary and metastatic melanoma samples according to immunohistochemical and molecular analyses

		Primary melanoma							Metastasis number 1							Metastasis number 2						
		IHC* data				Molecular data			IHC* data				Molecular data			IHC* data					Molecular data	
Patient	Age, years	Site	*BRAF*	*NRAS*	*c-KIT*	*BRAF*	*NRAS*	*c-KIT*	Site	*BRAF*	*NRAS*	*c-KIT*	*BRAF*	*NRAS*	*c-KIT*	Site	*BRAF*	*NRAS*	*c-KIT*	*BRAF*	*NRAS*	*c-KIT*
1	79	T	wt	wt	wt	wt	Q61L	wt	L	wt	wt	m, het	wt	Q61L	wt	SK	wt	wt	wt	wt	Q61L	wt
2	81	A	wt	wt	s, hom	wt	wt	wt	L	wt	wt	wt	wt	wt	wt							
3[a]	56	A	wt	wt	wt	wt	wt	wt	SK	wt	wt	wt	wt	wt	wt	L	wt	wt	wt	wt	wt	wt
4	73	E	s, hom	wt	wt	V600E	wt	wt	SK	s, hom	wt	wt	V600E	wt	wt							
5	54	T	m, het	wt	wt	wt	wt	wt	S	wt	wt	wt	V600K	wt	wt							
6	69	E	wt	w, het	wt	wt	wt	wt	L	wt	wt	wt	wt	wt	wt							
7	82	E	wt	s, hom	wt	wt	Q61R	wt	L	wt	s, hom	wt	wt	Q61R	wt							
8	80	T	wt	wt	wt	wt	Q61L	wt	L	wt	wt	wt	wt	Q61L	wt							
9	65	T	s, het	wt	wt	V600E	wt	wt	L	w, het	wt	wt	V600E	wt	wt							
10	70	E	s, hom	wt	wt	V600E	wt	wt	L	s, hom	wt	wt	V600E	wt	wt	B	s, hom	wt	m, het	V600E	wt	amp
11	57	E	s, hom	wt	wt	wt	wt	wt	L	m, het	wt	wt	wt	wt	wt							
12	70	H	wt	wt	wt	V600K	wt	wt	SK	wt	wt	wt	V600K	wt	wt	L	wt	wt	wt	V600K	wt	wt
13	57	T	s, hom	wt	wt	V600E	wt	wt	SK	s, hom	wt	wt	V600E	wt	wt							
14[b]	25	E	s, het	wt	m, het	V600E	wt	wt	L	s, hom	wt	m, het	V600E	wt	wt	L	s, hom	wt	wt	V600E	wt	wt
15	65	T	s, hom	wt	wt	V600E	wt	wt	L	w, het	wt	wt	V600E	wt	wt							
16	56	T	wt	wt	m, het	V600K	wt	amp	L	wt	wt	wt	wt	Q61L	wt							
17	45	E	wt	m, hom	wt	wt	wt	wt	L	wt	s, het	wt	wt	Q61R	wt	SK	wt	s, het	wt	wt	Q61R	wt
18	35	E	s, hom	wt	wt	V600E	wt	wt	L	s, het	wt	m, het	V600E	wt	wt							
19	52	H	s, hom	wt	m, het	V600E	wt	wt	V	wt	wt	m, het	wt	wt	wt							
20	63	E	wt	wt	wt	wt	wt	L802F	L	wt	wt	wt	wt	wt	wt							
21	58	H	wt	wt	m, het	V600K	wt	wt	SK	wt	wt	wt	V600K	wt	wt	V	wt	wt	wt	V600K	wt	wt
22	76	A	wt	wt	m, het	wt	wt	wt	L	wt	wt	m, het	wt	wt	wt							
23	66	E	wt	wt	wt	V600K	wt	amp	B	wt	wt	wt	V600K	wt	wt							
24	84	E	wt	wt	wt	wt	wt	wt	L	wt	wt	wt	wt	Q61L	wt							
25	76	H	wt	wt	s, hom	wt	wt	wt	SK	wt	wt	s, hom	wt	wt	wt							
26	69	T	wt	wt	wt	wt	Q61K	wt	L	m, het	wt	wt	V600E	wt	wt							
27	49	T	s, hom	wt	wt	V600E	wt	wt	L	s, hom	wt	wt	V600E	wt	wt							
28	44	T	s, hom	wt	wt	V600E	wt	wt	L	s, hom	wt	wt	V600E	wt	wt							
29	74	E	wt	s, hom	m, het	wt	Q61R	wt	SK	wt	s, het	wt	wt	Q61R	wt							
30	75	H	wt	s, hom	m, het	wt	Q61R	wt	V	wt	s, het	wt	wt	Q61R	wt							

[a]This patient also developed a third nodal metastasis that was wild-type for *BRAF* and *NRAS*, both at immunohistochemical (IHC) and molecular analysis. [b]This patient also developed a third skin metastasis that carried the $BRAF^{V600E}$ mutation both at IHC and molecular analysis.*Staining intensity (s: strong; m: moderate; w: weak) and distribution (het: heterogeneous; hom: homogeneous) are indicated for IHC positive cases.
A: acral site; amp: amplification (gene copy number ≥3); B: brain, E: extremities, H: head and neck; S: skin; V: visceral; L: lymph node; SK: skin; wt: wild type.

mutation ($BRAF^{V600K}$ in 2 cases and $BRAF^{V600E}$ in one case).

A significant association between mutational status and clinical characteristics of the primary melanoma was observed only for median age at melanoma onset (**Table III**). Patients with $BRAF^{V600}$ mutated melanomas were younger than those with *BRAF* wild-type melanomas (57 vs. 74 years, $p<0.01$), while *NRAS* mutations were more prevalent in older patients (77 vs. 60 years, $p<0.01$). A higher trend of *NRAS* mutations were found in primary melanomas showing CDS, as evaluated by histological solar elastosis ($p=0.06$).

An intra-patient concordance of *BRAF* mutational status between primary melanoma and related metastases was detected in 86.7% (26/30) of patients, with 9 (9/26, 34.7%) concordant for $BRAF^{V600E}$, 3 (3/26, %) for $BRAF^{V600K}$, and 14 (14/26, 53.8%) for the wild-type genotype (Table II and **Fig. 1**). Intra-patient *BRAF* molecular heterogeneity was observed in 13.3% (4/30) of patients. Notably, all patients (7/30, 23.3%) with multiple metastases showed a consistent *BRAF* mutational status between primary melanoma and all analysed metastatic tissues. Concerning *NRAS* mutational profile, a concordant *NRAS* mutational status was observed in 26 of 30 (86.7%) patients, with the majority (21/30, 70.0%) a wild-type genotype (Fig. 1). Among the 4 discordant cases (4/30, 13.3%), 3 (3/4, 75%) showed wild-type primaries and mutated metastases with the following genotypes Q61R, Q61L and Q61K (Table II). Concordance rate of *BRAF* and *NRAS* mutational status did not differ by sex, timing of metastases appearance or metastatic site (Table SII).

Regarding the single patient mutated for *c-KIT*, a discordant mutational status was observed between his primary and metastatic lesion.

Table III. Frequency of *BRAF* and *NRAS* mutations according to clinical and histopathological characteristics of patients and tumours

		BRAF			*NRAS*		
	All samples	Wild-type	Mutated	*p*-value	Wild-type	Mutated	*p*-value
Characteristics of patients	*n* = 30	*n* = 16	*n* = 14		*n* = 24	*n* = 6	
Sex, *n* (%)				0.16			0.71
Males	17 (56.7)	7 (43.7)	10 (71.4)		14 (58.3)	3 (50.0)	
Females	13 (43.3)	9 (56.3)	4 (28.6)		10 (41.7)	3 (50.0)	
Age at diagnosis, years, median (range)	65 (25–84)	74.5 (45–84)	57.5 (25–73)	**<0.01**	60.5 (25–84)	77 (69–82)	**<0.01**
Characteristics of primary tumours	*n* = 30	*n* = 16	*n* = 14		*n* = 24	*n* = 6	
Breslow thickness, mm, median (range)	4.2 (0.6–30)	3.8 (1.2–20.0)	4.9 (0.6–30.0)	0.91	5.7 (0.6–30)	2.7 (1.2–19)	0.23
Anatomical site, *n* (%)				0.28			0.69
Head/neck	5 (16.7)	2 (12.5)	3 (21.4)		4 (16.7)	1 (16.7)	
Trunk	10 (33.3)	4 (25)	6 (42.9)		7 (29.2)	3 (50.0)	
Extremities	12 (40.0)	7 (43.7)	5 (35.7)		10 (41.6)	2 (33.3)	
Acral	3 (10.0)	3 (18.8)	0		3 (12.5)	0	
Solar elastosis, *n* (%)				0.94			0.06
No	12 (40.0)	6 (37.5)	6 (42.9)		12 (50.0)	0	
Yes	18 (60.0)	10 (62.5)	8 (57.1)		12 (50.0)	6 (100.0)	
Naevus-associated melanom, *n* (%)a				0.71			0.64
No	19 (63.3)	11 (68.8)	8 (57.1)		16 (66.7)	3 (50.0)	
Yes	11 (36.7)	5 (31.2)	6 (42.9)		8 (33.3)	3 (50.0)	
Histological subtype, *n* (%)				0.20			0.60
Superficial spreading melanoma	7 (23.3)	2 (12.5)	5 (35.7)		5 (20.8)	2 (33.3)	
Nodular melanoma	23 (76.7)	14 (87.5)	9 (64.3)		19 (79.2)	4 (66.7)	
Ulceration, *n* (%)				0.71			0.52
No	10 (33.3)	6 (37.5)	4 (28.6)		9 (37.5)	1 (16.7)	
Yes	20 (66.7)	10 (62.5)	10 (71.4)		15 (62.5)	5 (83.3)	
Number of mitoses, median (range)	5 (0–24)	5.0 (0–24)	5.5 (0–23)	0.89	5.0 (0–23)	5.0 (1–24)	0.73
Characteristics of metastasis	*n* = 39	*n* = 20	*n* = 19		*n* = 29	*n* = 10	
Anatomical site, *n* (%)				0.42			0.85
Brain	2 (5.1)	0	2 (10.5)		2 (6.9)	0	
Lymph node	23 (59.0)	13 (65.0)	10 (52.6)		17 (58.6)	6 (60.0)	
Skin	11 (28.2)	5 (25.0)	6 (31.6)		8 (27.6)	3 (30.0)	
Visceral	3 (7.7)	2 (10.0)	1 (5.3)		2 (6.9)	1 (10.0)	

Considering the overall somatic profile of all *BRAF/NRAS/c-KIT* genes, intra-patient heterogeneity was present in 23.3% (7/30) of patients (6 with 1 metastasis and 1 with 2 metastases). Discordance rates were not associated with sex, synchronicity or anatomical sites of metastasis (Table SII[1]).

Immunohistochemistry

A total of 26 (26/69, 37.7%) samples showed positive immunostaining with anti-BRAF^{V600E} VE1 antibody with homogenous staining in 19 of 26 (73.1%) tissues. Staining intensity was strong in 22 tumours (22/26, 84.6%), moderate in 3 (11.5%) and weak in 1 (1/26, 3.9%). Overall, primary melanomas (12/30, 40.0%) and metastatic lesions (14/39, 35.9%) showed similar frequency of *BRAF*^{V600E} staining ($p = 0.46$). Regarding *NRAS*, 10 tumour tissues (10/69, 14.5%) were positive for *NRAS*^{Q61R} SP174 immunostaining and 59 were negative (59/69, 85.5%). Staining intensity was strong in 8 tumours (8/10, 80.0%), and moderate or weak in 1 (1/10, 10.0%) each. No significant difference was observed in the frequency of *NRAS*^{Q61R} positive staining between the groups of primary melanomas (5/30, 16.7%) and metastatic lesions (5/39, 12.8%) ($p = 0.45$).

All 11 melanomas arising in association with a melanocytic naevus showed concordance for *BRAF* and *NRAS* between melanoma cells and naevus cells, with 5 of them (5/11, 45.4%) showing positive immunostaining for *BRAF*^{V600E} and 3 (3/11, 27.3%) for *NRAS*^{Q61R}; the remaining cases were negative for both mutations (**Fig. 2**).

Sixteen tumours tissues (16/69, 23.2%) showed positive CD117/c-KIT expression, with 13 cases presenting a moderate and 3 cases a strong immunostaining. A trend for a higher prevalence of positivity was observed in the group of primary melanomas (9/30, 30.0%) than in metastatic samples (7/39, 17.9%), although not statistically significant ($p = 0.06$).

Considering the mutational findings obtained by IHC, the intra-patient *BRAF*^{V600E} concordance between primary lesions and related metastases was present in 27/30 (90.0%) patients, including 10 (10/30, 33.3%) with *BRAF*^{V600E}-positive lesions and 17 (17/30, 56.7%) negative. Intra-patient *BRAF*^{V600E} heterogeneity was observed in 10.0% (3/30) of patients (Fig. 2). For *NRAS*^{Q61R}, the majority of patients (29/30, 96.6%) showed an intra-patient concordant immunostaining between the primary lesion and related metastases, being 4 (4/30, 13.3%) consistent for *NRAS*^{Q61R} positivity and 25 (25/30, 83.3%) for negative staining. A discrepant *NRAS*^{Q61R} staining was observed in 1 patient (3.3%). Finally, intra-patient concordance of CD117/c-KIT expression between primary lesions and related metastases was present in 22/30 (73.3%) patients, including 3 (3/30, 10.0%) with positive and 19 (18/30, 63.3%) with negative tissues (Table II). Three patients with multiple metastases showed heterogeneity among their tumour tissues.

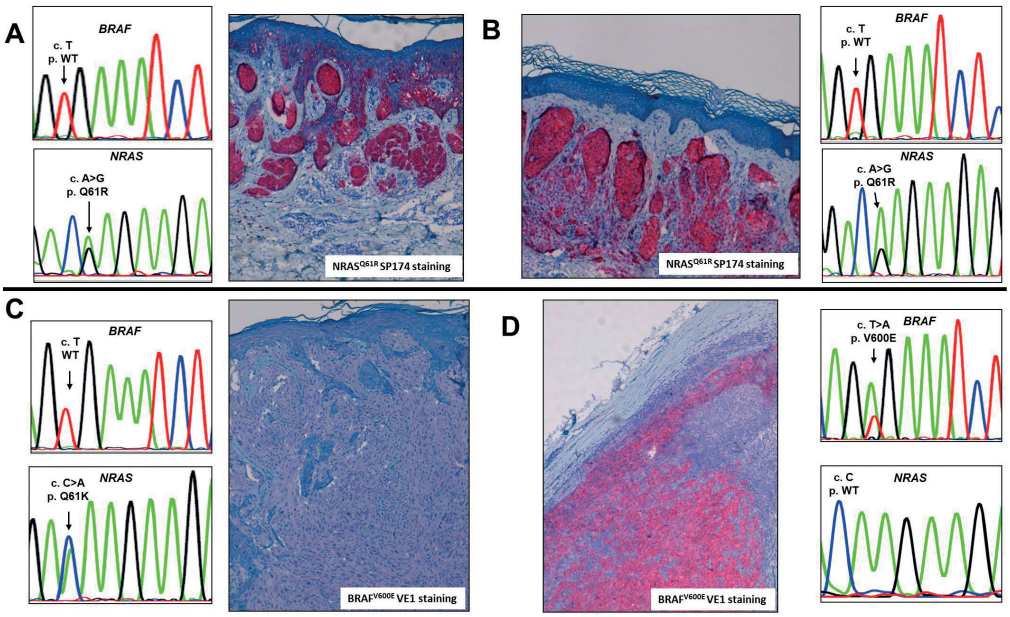

Fig. 1. Illustrative cases of *BRAF* and *NRAS* mutational status in primary melanoma and related metastasis by sequencing and immunohistochemistry. (A, B) Intra-patient *NRAS* concordance. (A) Primary melanoma and (B) cutaneous metastasis: positive *NRAS*^{Q61R} SP174 immunostaining and *NRAS*^{Q61R} mutation sequencing in both lesions. Magnification ×10. (C, D) Intra-patient *BRAF* and *NRAS* heterogeneity. (C) Primary melanoma: negative *BRAF*^{V600E} VE1 immunostaining and *BRAF* wild-type on mutation sequencing. Identification of the *NRAS*^{Q61K} mutation. (D) Metastatic lymph node: positive *BRAF*^{V600E} VE1 immunostaining and *BRAF*^{V600E} mutation sequencing. Magnification ×10.

Correlation between molecular analysis and immunohistochemistry

BRAF^{V600E} VE1 immunostaining was consistent with *BRAF* molecular findings in 56 of 59 (94.9%) tissues, with 23 (23/56, 41.1%) *BRAF*^{V600E} mutated and 33 (34/56, 60.7%) wild-type (Table III). The 10 (10/69, 14.5%) samples harbouring a *BRAF*^{V600K} mutation were indeed negative on IHC VE1 staining and were not included in this analysis. Discrepant findings were found in 3 of 59 (5.1%) samples: all cases were positive for immunostaining, but wild-type on molecular testing (both Sanger sequencing and competitive allele TaqMan™ PCR). Two discordant (2/3, 66.7%) cases (1 primary melanoma and 1 metastasis) had a moderate and heterogeneous *BRAF*^{V600E} VE1 staining pattern, while the remaining sample (1 primary melanoma) presented a strong positive and homogeneous staining. *BRAF*^{V600E} VE1 antibody sensitivity was 100.0%, specificity 91.7%, accuracy 94.9%. Overall, the agreement between molecular testing and IHC was "almost perfect" (Cohen's kappa = 0.90; $p<0.001$).

SP174 *NRAS*^{Q61R} immunostaining and *NRAS* molecular analysis showed a high rate of consistency (60 of 61 cases, 98.4%), with 8 (8/60, 13.3%) tissues carrying the *NRAS*^{Q61R} mutation and 52 (52/60, 86.7%) the wild-type genotype. Cases carrying other *NRAS*^{Q61} mutations showed no IHC SP174 staining and were not included in the analysis. Only one primary melanoma resulted positive for IHC *NRAS*^{Q61R} with weak and heterogeneous staining pattern, but wild-type at the molecular analysis. The sensitivity of the SP174 NRAS^{Q61R} antibody was 100.0%, specificity 96.2%, and accuracy 96.7%. Overall, the agreement between molecular testing and IHC was "almost perfect" (Cohen's kappa = 0.87; $p<0.001$).

Regarding *c-KIT* gene, no mutation was found in all 16 samples with a high level of CD117/c-KIT by IHC, while 2 of the 3 (66.7%) cases with *c-KIT* gene amplification showed increased expression of CD117/c-KIT.

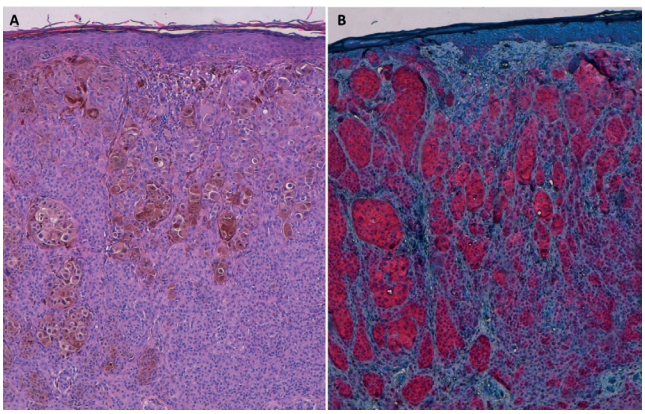

Fig. 2. *BRAF*^{V600E} VE1 staining in naevus-associated melanoma. Concordant positive *BRAF*^{V600E} in melanoma and naevus. (A) Haematoxylin and eosin staining, (B) *BRAF*^{V600E} VE1 staining. Magnification ×20.

DISCUSSION

This study identified *BRAF* mutations in 46.7% of primary melanomas and in 48.7% of metastases and *NRAS* mutations in 20% and in 25.6%, respectively. The intra-patient molecular heterogeneity between primary melanoma and related metastases was detected in 13.3% of patients for both *BRAF* and *NRAS* genes and was not associated with clinico-pathological characteristics of melanoma or metastases. We demonstrated consistency of *BRAF*^{V600E} and *NRAS*^{Q61R} mutational findings obtained by molecular analysis and IHC immunostaining for both overall mutation frequencies and intra-patient heterogeneity with an "almost perfect" agreement.

Recently, 4 different molecular melanoma subtypes were proposed based on the type of driver MAPK activa-

ting gene mutation, i.e. *BRAF* (35–50% of cases), *NRAS* (10–25%), *NF1* (~ 15%) mutated and triple-wild-type melanomas (~10%) including *c-KIT* mutated lesions (32). These oncogenic alterations have been associated with different clinico-pathological aspects of patients or tumours, such as age, anatomical site and degree of cumulative sun exposure (CSD or not CSD) (7).

Intra-patient molecular heterogeneity between primary melanoma and related metastases has important implications in clinical practice when metastatic patients with discordant lesions need to be treated with targeted therapy. In addition, comparison between primary lesions and related metastases can provide insights into the processes involved in metastatic progression. We observed an intra-patient discrepancy in *BRAF* mutations between primary and metastatic lesions in 13.3% of patients, as evaluated by molecular methods, in line with the discordance rate of 13.4% and 15.5% reported in 2 recent meta-analyses (16, 27). A true biological manifestation of tumour heterogeneity, but also technical issues (molecular-based methods compared with IHC-based) have been hypothesized to explain these discrepancies (16, 27, 28). A higher mutational discordance rate has been reported with the increasing number of metastases, i.e. 8% in patients with one metastasis, 18% in patients with 2 metastases, and 20% in patients with 3 metastases (16). All our patients with multiple metastases (23.3%) showed a consistent *BRAF* status between primary melanoma and all metastatic tissues.

Regarding *NRAS*, the intra-patient discordance rate was reported to range from 3% to 14.3% of cases in few small studies (24, 26, 29), while no discrepancy was found in one study (17). A 13.3% discordant rate was observed in our patients, with the majority of them acquiring the *NRAS* mutation over time as part of disease progression.

Controversial results were reported for the rate of intra-patient *BRAF* or *NRAS* discrepancies according to the site of metastasis, with a suggested, but not confirmed, higher rate of discordance between primary melanoma and visceral metastases compared with lymph node metastases (17). The discrepancy rate for locoregional lymphatic metastases was indeed reported to range from 9.2% to 38% across 4 previous studies (19, 21, 26, 33), while for visceral metastases, including brain, from 13% to 50% (26, 33, 34). In the current study no differences were observed in the heterogeneity rate between primary melanoma and lymph node or visceral metastatic sites.

The intra-patient heterogeneity between primary melanoma and related metastases has been hypothesized to be due to the specific detection method, with a higher rate of heterogeneity for molecular methods than for IHC approaches (12, 19, 23, 25, 27, 28). Therefore, we compared $BRAF^{V600E}$ and $NRAS^{Q61R}$ mutations using molecular methods (Sanger sequencing and allele-specific Taqman™ assays) and IHC. We observed very consistent findings between immunostaining and molecular methods for $BRAF^{V600E}$ mutation, as detected in 95% of our samples, and for $NRAS^{Q61R}$ found in 98% of cases. Interpretation issues have been reported for weak and moderately stained lesions, as in 3 of our 4 discordant cases, since they have been considered either positive or negative in the literature, thus suggesting that caution is necessary in case of unclear staining (13, 35). In addition, a rare VE1 antibody cross-reactivity with an unknown epitope may also be a possible explanation for false-positive staining (36, 37). Overall, in our cases the intra-patient heterogeneity between primary melanoma and metastatic tissues does not seem to be attributable to methodological aspects, since it was similar for IHC and molecular methods, thus possibly reflecting the true biological heterogeneity during melanoma progression.

Few small studies have investigated the concordance rate of $BRAF^{V600E}$ and $NRAS^{Q61R}$ mutations between naevus and melanoma in naevus-associated melanomas (14, 38–40). For *BRAF* gene, a concordance rate varying from 75% to 100% was reported in 4 studies (14, 38–40) and for *NRAS*, 91% of melanomas and associated naevi were concordant in one study (39). All 11 naevus-associated melanomas in our series showed a concordant of $BRAF^{V600E}$ and $NRAS^{Q61R}$ status between melanoma and naevus cells by immunostaining.

This study has a few limitations. *NF1* mutational analysis was not performed, since the clinical significance of *NF1* mutations in melanoma is unknown and the interest as a potential therapeutic target is currently scarce. In addition, this study did not have adequate statistical power to evaluate intra-patient heterogeneity of *c-KIT* mutations, probably due to the low prevalence of acral lentiginous and mucosal melanoma subtypes. Finally, our results on intra-patient heterogeneity are mainly referred to lymph node metastasis due to the low number of visceral metastasis in our sample that might have underestimated the overall discordance rate. However, the evaluation of the discordance rate between primary melanoma and nodal metastases in disease-free patients is nowadays important for administration of targeted therapies in the adjuvant setting.

These findings confirm that a relevant intra-patient heterogeneity between primary melanoma and related metastases exists independently of the technical approach, thus supporting the polyclonal model of melanoma progression. In addition, IHC and molecular methods provided highly consistent results in the detection of $BRAF^{V600E}$ and $NRAS^{Q61R}$ mutations, supporting IHC as a rapid and cost-effective screening method in melanoma, although a combined approach is necessary in cases with negative or doubtful immunostaining.

The authors have no conflicts of interest to declare.

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